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13. ABSTRACT *(Maximum 200 Words)*

Urinary tract infections (UTIs), generally caused by Escherichia coli or Staphylococcus saprophyticus, are extremely common among young women. Although UTIs can be treated, we currently lack effective means to prevent frequently UTIs, which occur in 25% of women with first UTI. A necessary prerequisite to UTI is adherence of uropathogens to the vaginal and bladder epithelium. This report describes the third year of progress in a project that defines uropathogenic E. coli and S. saprophyticus-binding glycosphingolipids (GSLs) in the vaginal and bladder epithelium, shown in preliminary studies to function as bacterial receptors, as a prerequisite to the rational design of new agents that will prevent colonization and infection in women. Key progress includes: (a) establishment of in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells; (b) characterization of GSLs expressed by these epithelial cells and of bacterial adherence to them; (c) studies of the effects of exogenous estrogen on GSL expression and bacterial adherence to each of these in vitro model systems; (d) cloning of a potential human α1-4Galactosyltransferase; and (e) enzymatic synthesis of globoseries based GSL compounds.

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9/29/99 Ann E Stapleton MD

PI - Signature

Date

4. TABLE OF CONTENTS

5. INTRODUCTION

a. Overview

The overall purpose of this project is to investigate interactions between bacteria which commonly cause urinary tract infection (UTI) and their cognate host cell receptors in the vaginal and bladder epithelium in order to design novel, non-antibiotic methods for preventing UTIs. The project is focused on studying the two most common uropathogens causing UTI in young women, Escherichia coli and Staphylococcus saprophyticus, as well as their interactions with glycosphingolipids (GSLs) on the cell surface of the bladder and vagina. In the first three years of this project, we will define the key GSLs on the eukaryotic cell surface that uropathogenic bacteria use for attachment and then in the last two years, we will take advantage of new biochemical techniques using carbohydrate mimetics to design UTI prevention methods that avoid the induction of antimicrobial resistance. This report describes progress made in the third of four years of this grant.

b. Background presented in original proposal

Acute uncomplicated UTIs caused by E. coli and S. saprophyticus occur in an estimated 7 million young women each year at an annual cost for diagnosis and treatment exceeding one billion dollars. Over half of all women have had a bacterial UTI by their late 20's and approximately 20% of women with UTI suffer very frequent (≥ 3/year) recurrences (1). Nonetheless, the only currently available preventive modality for these recurrent infections is antimicrobial prophylaxis. Though effective, antimicrobial prophylaxis may promote the emergence of drug-resistant strains (1). In addition, women typically revert to having frequent recurrences once prophylaxis ceases and little is known about why some women suffer frequent recurrences of UTI, since this phenomenon cannot usually be explained by underlying functional or anatomic abnormalities of the urinary tract (1). The interaction of infecting bacterial strains with the women's epithelial cells appears to be a critical point in the infectious process that determines host susceptibility, in particular the availability and nature of host cell bacterial ligands such as GSLs (2-4).

GSLs are important components of the glycocalyx surrounding mammalian cells and consist of an oligosaccharide moiety exposed on the cell surface, to which organisms attach, covalently linked to a lipid portion embedded in the outer leaflet of the plasma membrane. They serve as eukaryotic cell adhesion sites for many pathogens and their toxins, including E. coli, Pseudomonas aeruginosa, Helicobacter pylori, HIV, parvovirus, rotavirus, cholera toxin, verotoxin of E. coli 0157, and others (5-9). Based on the structures of their carbohydrate components, they are grouped into families, such as the lacto- and neolacto-series, the globoseries, and the ganglioseries GSLs. GSLs are synthesized by the sequential action of glycosyltransferases, many of which are tissue-specific and/or genetically determined (10). Thus, GSLs on the cell surface play an important role both in determining tissue tropism and an individual host's susceptibility to specific infectious diseases.

Among uropathogenic E. coli, isolates expressing the pap-encoded family of adhesins are significantly overrepresented among strains collected from patients with UTI as compared with fecal isolates from patients without UTI (11). The GSL receptors for these adhesins in the kidney are the globoseries GSL family that contain a minimal receptor consisting of a

galactose α-1-4 galactose moiety (11). Although S. saprophyticus is the second most common cause of UTI, to our knowledge, we are the first to actually investigate whether it binds to GSLs. In our grant proposal, we showed preliminary data demonstrating that the wild-type S. saprophyticus strain ST352 binds asialo GM1 (ASGM1), a neutral ganglioseries GSL and that other wild-type S. saprophyticus isolates bind to ASGM1 and/or structurally-related ganglioseries GSLs. Paradoxically, while globoseries GSLs have been identified in kidney tissue and vaginal epithelium, the bladder has been little studied with respect to GSLs, despite the fact that it is the most common site of UTI. Previous studies of GSLs in native bladder tissues have focused on oncogenesis or development and have not included GSLs that are directly involved in adhesion of uropathogens (ganglioseries and globoseries GSLs). Of note, other urogenital pathogens, including C. albicans, C. trachomatis, N. gonorrhoeae, have also been reported to bind to ASGM-1 and it is possible that a single class of inhibitors could prevent adherence and infection with all of these agents.

c. New background information: culture and characterization of vaginal epithelial cells

The native vaginal epithelium in vivo and in vitro

As a mucosal surface, the vagina serves as a barrier to local infection, but since it is situated at the entrance to the upper genital tract, it also serves protective functions. It is a non-keratinized, stratified squamous epithelium lacking glands. Based on cellular morphology by light microscopy, the epithelium is divided into several layers: the basal/proliferative, parabasal, intermediate, and superficial layers. A complex ecosystem exists within the vagina of post-menarchal women, consisting of at least 50 species of bacteria, but dominated by lactobacilli (2, 7, 18). 85% of the total bacterial counts in the vaginal fluid, typically 10^8 to 10^9, are members of this genus (2, 39). Clinical observations suggest a protective effect when lactobacilli dominate the vaginal flora. The risk of acquiring vaginal and cervical pathogens such as C. albicans, C. trachomatis, and bacterial vaginosis-associated organisms is reduced in women with lactobacilli predominating in their vaginal flora (34). Conversely, increased vaginal colonization with E. coli in young, otherwise healthy women is associated with increased risk of UTI (45), and during pregnancy, colonization with E. coli is associated with preterm birth and other perinatal complications (18, 34).

The onset of menopause in many women is associated with thinning of the vaginal mucosa and a change in the vaginal cytology to basal and parabasal morphologies. There is a loss of lactobacillus colonization and an increase in colonization with potential pathogens such as E. coli, Group B streptococcus, and enterococci (38) (Fihn et al, unpublished data). The use of topical hormone replacement therapy may result in a restoration of mature, superficial morphology of the vaginal cytology smear and a lactobacillus-dominant flora (38). However, perhaps because of the paucity of in vitro model systems, there have been few studies of the effects of exogenous sex hormones on nearly any aspect of the basic biology of the vaginal epithelium, including markers of differentiation, bacterial attachment and colonization with normal flora. Clinically, the application of certain intravaginal products such as spermicides often results in a reduction or loss of a Lactobacillus-dominated vaginal flora and an increased risk of UTI (20, 21). Prospective clinical studies of other potential effects of such products on the vaginal ecosystem and on colonization and infection with potential urinary and genital pathogens are ongoing. We postulate that further investigations of such

questions under more controlled, in vitro conditions would be ideally conducted using a model system such as that described in this proposal.

Potential mechanisms of the apparently protective effect of lactobacilli are only partially understood. The normal vaginal ecosystem in menstruating women is characterized by a pH in the range of 3.8 to 4.7 (31, 33), which is inhibitory to the growth of many pathogens. The lactic acid produced by lactobacilli is thought to be a major contributor to maintaining this pH range (31, 33). Hydrogen peroxide (H_2O_2), produced by 85-95% of lactobacilli in normal flora of pre-menopausal women (31, 33) is important because it has a microbicidal effect for certain pathogens, including E. coli (27). Among the various members of the Lactobacillus genus present in the human vagina, L. crispatus appear to be particularly important in the production of H_2O_2 (44). Adherence of lactobacilli to the vaginal epithelium has also been postulated to have a protective effect on this mucosa, perhaps preventing the adherence of pathogens (8), through steric hindrance and/or competition for receptors (40). Mechanisms by which lactobacilli adhere to VECs are largely unknown and their cognate receptors on this epithelium have also been scarcely investigated. One report described binding of Lactobacilli to globoseries GSLs (5), suggesting that these organisms could directly compete with uropathogenic E. coli for binding to vaginal epithelium. We have conducted one of the few surveys of Lactobacillus adherence to native VECs and found that the degree of adherence to exfoliated VECs is quite variable when individual Lactobacillus strains are compared (1).

Keratins in vaginal tissue

The expression of cytokeratins and of involucrin has been more thoroughly studied in other stratified squamous epithelium such as the mouth. A review of these studies as well as studies of native human and mouse vaginal tissue suggests patterns of keratin expression correlating with specific cell layers and with morphological degrees of differentiation have been developed. K19, generally associated with epithelial proliferation, has been found in basal layers of human vaginal epithelium (14). K13 has been used as a marker of suprabasal cell layers and thus of differentiation (42, 43). In the mouse, K14 is found in all layers of the epithelium and K1 was not expressed in undifferentiated epithelium but was upregulated in response to exogenous estrogen (15). Human vagina stained in all layers for K10, usually associated with the epidermis in the skin (14). Involucrin, a marker of terminal differentiation, was only found in the superficial layers of human vaginal epithelium (12).

d. Brief summary of preliminary data presented in original proposal

Our original proposal presented preliminary data demonstrating that primary cultures of human bladder epithelial cells are a promising model system for the study of bladder GSLs in the pathogenesis of UTI caused by E. coli or S. saprophyticus. Specifically, we showed that these cell cultures appear to express globoseries GSLs, the host cell binding ligand for an important class of uropathogenic E. coli, those expressing pap-encoded adhesins. We previously showed that SGG and other globoseries GSLs are surface exposed in human kidney tissues and exfoliated vaginal epithelial cells (2). In our preliminary immunocytology experiments with primary cultures of human uroepithelium, the cells demonstrated bright immunofluorescent staining with MAb ID4 directed against SGG, suggested that this epitope is also surface exposed on these bladder cells. We also showed that S. saprophyticus, the second most common cause of UTI in young women, binds to ganglioseries GSLs, especially

ASGM1 and ASGM2. In addition, we demonstrated the presence of ASGM1 among GSLs extracted from human kidney and the surface exposure of this epitope in kidney sections in specific histological areas where bacteria also adhere. Another ganglioseries GSL, GM1, was identified among GSLs extracted from human kidney tissues and vaginal epithelial cells. ASGM1 appears to be surface exposed on both kidney and cultured primary bladder cell surfaces, as shown by positive immunofluorescent staining with MAb TKH-7, directed against ASGM1.

d. Originally proposed hypotheses

The original hypotheses of this project have been supported by data obtained during the first two years of the grant and thus remain unchanged, as listed below. The overall goal of this project remains to define the key eukaryotic cell surface GSLs that are used by uropathogenic bacteria for attachment and then to take advantage of new biochemical techniques utilizing carbohydrate mimetics to design novel means for preventing UTIs that avoid the use of antimicrobials.

(1) We hypothesize that globoseries and ganglioseries GSLs are present in primary cultures of bladder transitional epithelium and vaginal epithelium and serve as binding sites for E. coli and S. saprophyticus, respectively.

(2) We hypothesize that the GSLs identified in the first hypothesis are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

(3) We hypothesize that carbohydrate mimetic and synthesis techniques can be used to design high-affinity inhibitors of E. coli and S. saprophyticus binding to vaginal and bladder transitional epithelium.

e. Original technical objectives

During the third year of funding for this project, we did not alter any of our technical objectives, but we increased the emphasis on certain aspects of our objectives. We expanded previously established collaborations and developed new ones, some of which promise to open new areas of inquiry in the coming year. For some of the objectives, we used slightly different but more efficient technical approaches. These changes are discussed in greater detail below. Our technical objectives are as follows:

(1) We will extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional epithelium and vaginal epithelium, according to the following sequence: (a) purify the GSLs using high-performance liquid chromatography (HPLC); (b) identify bacteria-binding GSLs by overlaying radiolabeled isolates of E. coli and S. saprophyticus on these GSLs separated on high-performance thin-layer chromatography (HPTLC); (c) confirm the identities of these GSLs using specific monoclonal antibodies (MAbs) directed against the GSLs in HPTLC immunostaining assays; and (d) perform carbohydrate structural analysis on the bacteria-binding GSLs.

(2) To demonstrate that the GSLs identified in Hypothesis 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally

relevant for E. coli and S. saprophyticus attachment and infection, we will: (a) test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures; (b) utilize immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs; (c) repeat 2a and 2b after pretreatment of the cell cultures with an inhibitor of GSL receptor synthesis; and (d) repeat 2a after pretreatment of the cell cultures with the MAbs directed against relevant GSLs.

(3) We will use carbohydrate mimetic techniques to design inhibitors of bacterial adherence, focusing initially on the interaction of E. coli with sialosyl galactosyl globoside (SGG, a GSL to which E. coli binds with high affinity; see preliminary data). We will test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays, as described in the second objective.

6. BODY OF REPORT

A. Overview

1. Review of changes and opportunities occurring in the first two years of funding:

(1) Establishment of a new collaboration with Dr. Steven Levery of the Complex Carbohydrate Research Center (CCRC) of The University of Georgia, Athens: We continue to work with Dr. Levery for assistance in structural characterization of GSLs (see results).

(2) Establishment of a collaboration with Drs. M. Juliana McElrath and Florian Hladik of the Fred Hutchison Cancer Research Institute and the University of Washington: Though this project has ended, but the technical aspects and opportunities associated with this work has been replaced this new collaborations involving members of the Department of Obstetrics and Gynecology. (see below).

(3) Vaginal epithelial cell cultures: This aspect of the project has become a major focus of the project in the past year, through expansion of technical knowledge, development of new collaborations and protocols for tissue acquisition, and the development of new hypotheses. These are detailed below.

(4). New approaches taken by Dr. Stroud: Dr. Stroud has further expanded new approaches developed in the last funding period for accomplishing Technical Objective 3, detailed below.

2. New opportunities and approaches during the third year of funding:

1. Bladder cell cultures: we have begun collaborating with Dr. Richard Grady, Assistant Professor of Urology at the University of Washington and Children's Hospital and Regional Medical Center. Dr. Grady has assisted us in obtaining urothelial tissue and in augmenting our culture stocks.

2. Meetings with collaborators based outside Seattle:

a. Dr. Atala: Dr. Stapleton met with Dr. Atala in May 1999 and plans another meeting in November 1999.

b. Dr. Levery: Dr. Stroud met with Dr. Levery during several visits by Dr. Levery to Seattle.

c. Dr. Toyokuni: Dr. Stroud will meet with Dr. Toyokuni in November at a West Coast glycobiology meeting this fall.

B. Original Statement of Work

The original technical objectives set for all four years of funding are listed below. Tasks on which we have made progress are noted in bold italic.

Technical Objective 1: Extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional and vaginal epithelium.

Task 1:	***Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells***
Task 2:	***Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures***
Task 3:	***Months 7 to 12: bacterial overlay assays***
Task 4:	***Months 7 to 12: immunostaining assays***
Task 5:	***Months 13 to 24: carbohydrate structural analysis***
Task 6:	***Months 25 to 36: data analysis and publication***

Technical Objective 2: Demonstrate that the GSLs identified in Technical Objective 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

Task 1:	***Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures***
Task 2:	***Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs***
Task 3:	***Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays***
Task 4:	***Months 24 to 36: MAb pre-treatment, followed by GSL extraction and quantification and bacterial adherence assays***
Task 5:	***Months 30 to 36: data analysis and publication***

Technical Objective 3: Use carbohydrate mimetic techniques to design inhibitors of bacteria adherence, focusing initially on the interaction of *E. coli* with SGG; test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays.

Task 1:	***Months 24 to 42: synthesis of linear mimetic compounds***
Task 2:	***Months 27 to 42: structural analysis of resulting compounds (procedures to begin as each new compound is prepared)***
Task 3:	Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial overlay assays, focusing on SGG as the target GSL to which binding should be inhibited
Task 4:	Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial adherence assays, using primary cultures of bladder and vaginal epithelial cells
Task 5:	Months 30 to 48: synthesis of multivalent structures from linear structures that are effective in Tasks 3 and 4
Task 6:	Months 40 to 48: repeating Tasks 3 and 4 using multivalent compounds
Task 7:	***Months 40 to 48: data analysis and publication***

C. Details of Progress

1. Technical Objective 1

a. Task 1, Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells

1. Experimental methods, assumptions and procedure

Primary cultures of human bladder epithelial cells were provided by Dr. Anthony Atala and maintained in serum free keratinocyte media using standard tissue culture techniques, as in our preliminary studies and as he has described (13). Briefly, cells were maintained in serum- and antibiotic-free keratinocyte medium and passed at 70% confluence in a ratio of 1:4 or 1:6 (13). Each time cells were passed, the number of flasks were maximally expanded to prepare cells for the purification of GSLs of interest. This procedure was continued with each cell sample until senescence was noted, usually at about passage 12.

In addition, we established primary cultures of bladder epithelial cells in our own facility, with the assistance of Dr. Richard Grady. To accomplish this, we adapted the methods developed for culture of primary vaginal epithelial cells, with one change. Specimens from bladder epithelium are generally processed in the operating room, rather than transporting them back to our facility first. Cultures are maintained as described above.

The collaboration we established with Drs. McElrath and Hladik during the first two years of funding resulted in our establishing a technique of maintaining primary cultures of vaginal epithelial cells in laboratory. The parent project for this collaboration has since ended, thus we replaced it with a project of our own. We added several new collaborators, listed below.

Details of how these collaborations have assisted us in obtaining data this past year or on how next year's work will be facilitated by planned collaborative studies are noted in the results and recommendations sections for the relevant tasks below.

(1) **David Eschenbach, MD**, Professor of Obstetrics and Gynecology, University of Washington, was a key contact person in the original project and has been a collaborator of ours on other projects for many years. During the past year of funding, he has provided vaginal tissue biopsy specimens , and with his assistance, we recently developed an independent protocol for tissue acquisition. Along with Dr. Patton (see below), he has consulted on issues regarding investigating estrogen and progesterone stimulation of our primary vaginal epithelial cells lines, in order to better approximate the biology of the vaginal epithelium in young, premenopausal women. This was the focus of much of our efforts in primary cell culture this past year.

(2) **Dorothy Patton, PhD**, Professor of Obstetrics and Gynecology and Adjunct Professor, Biological Structure and Ophthalmology, University of Washington. Dr. Patton has assisted us with issues specific to culturing vaginal epithelium and with immunohistostaining these tissues. Since she has years of prior experience with genital tissue-derived cultured cells, she has assisted us with assessing the effects of hormonally stimulating our vaginal epithelial cell cultures.

(3) **Beverly Dale, PhD**, Professor of Oral Biology and Adjunct Professor, Medicine (Dermatology), Periodontics, and Biochemistry at the University of Washington. Dr. Dale will assist us in determining markers of epithelial differentiation and in assessing epithelial morphological changes in response to estrogen during the final year of funding.

2. Results and discussion

Primary bladder epithelial cells: As noted above, we began cultivation of bladder epithelial cells from samples obtained through a collaborator at Children's Hospital in Seattle, to augment our work with Dr. Atala. We have maintained our collaboration with Dr. Atala in order to compare the results obtained in our hands, using his techniques, with cell established in his laboratory, with his established methods. This funding year, we have processed 22 samples, with the following outcomes:

Number of samples	Outcome
9	Cell line established; pellets obtained for GSL extraction; stocks of these lines established in liquid nitrogen
3	Cell line established; pellets obtained for GSL extraction
3	Tissue never attached and was discarded
2	samples were combined with one another by accident and were discarded
1	Contaminated before establishment
4	recently established cultures; results pending

Primary vaginal epithelial cells: During the first two years of funding, we processed a total of 20 samples in attempts to establish lines of primary vaginal epithelial cells. With time, our skills in all aspects of these techniques improved and a larger proportion of our efforts were

successful. In the past year of funding, we processed an additional 14 samples, with the following results:

Number of samples	Outcome
7	Cell line established; pellets obtained for GSL extraction; stocks of these lines established in liquid nitrogen
3	Cell line established, but insufficient material for storage of stocks
3	Contaminated before establishment
1	error in transport to lab

With respect to five of these primary vaginal epithelial cells lines for which pellets of cellular material for GSL extraction were saved: (a) three are greater than 0.5 ml in size; and (2) for two lines, extensive characterization of their response to estrogen stimulation has been performed (see below).

Details of technical progress in culture efforts are as follows:

a. General: Issues regarding seeding, avoidance of contamination, and eliminating fibroblast contamination: these techniques are all routine in the laboratory now. We have also further adapted our methods for large surface area cultivation to obtain larger cell pellets for GSL characterization.

b. Cryopreservation: Our collection of cryopreserved cells has been further augmented, and methods for re-seeding frozen cultures have been maximized. We have been able to revive cultures preserved over one year ago.

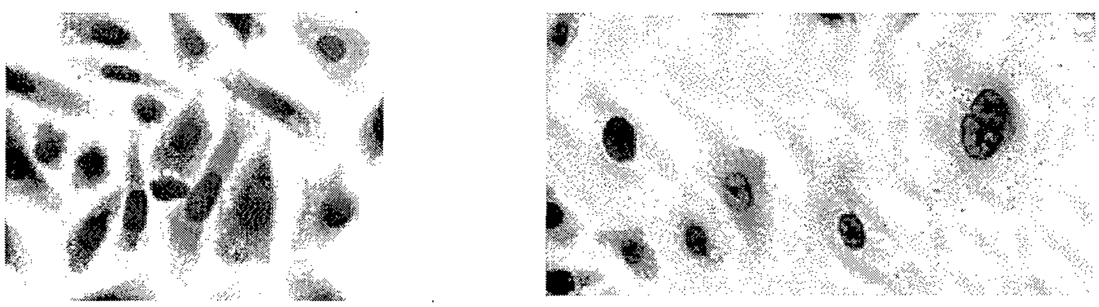
c. Characterization of cultured vaginal epithelial cells

We have continued investigating our efforts at characterizing the cultured VECs and have conducted pilot experiments to investigate the effects of estrogen and of serum on the differentiation of the cells. We have studied the cells' morphology under light microscopy, expanded our investigations of adherence characteristics of the cells, and begun studying the effects of estrogen on GSL expression. Dr. Patton, an expert in the histopathology of gynecological tissues, has continued to work closely with us on evaluating our cultures morphologically. We have recently set up a new collaboration with Dr. Beverly Dale, an expert in epithelial differentiation and in the biology of the oral epithelium, which appears to be biologically similar to the vaginal epithelium. Other pilot experiments included (1) very preliminary experiments on progesterone stimulation of the cultured cells; (2) growth of VECs unattached in media and studies of adherence to these cells, in order to compare data with our previous work with exfoliated native VECs; and (3) adherence experiments using Lactobacillus strains derived from the normal vaginal flora. Data from the cell characterization studies are presented below, while results of adherence experiments and GSL characterization studies are presented in the relevant sections.

Our initial goals in studying the effect of estrogen on cultured VECs was to establish concentration parameters for morphological changes and toxicity. Based on in vivo observations, we hypothesized that exogenous estrogen (17-ß-estradiol) would cause

"maturation" of the VECs, producing changes in morphology consistent with suprabasal or even superficial cells in vivo. We also investigated the effect of phenol red normally present as a pH indicator in keratinocyte media, because of a report of weak estrogenic effects attributable to phenol red when contained in media used on estrogen-responsive cells (2). Thus, we investigated the effects on morphology, adherence, and GSL expression of cells grown in a specially prepared serum-free medium without the phenol red indicator (PRF-SFM; Gibco). The phenol red concentration in the ordinary keratinocyte-SFM medium (SFM) was 3.1 μM. This medium was critical for embarking upon the estrogen stimulation pilot studies that were proposed in last year's annual report. Lastly, because of experience with cultured primary transitional epithelial cells, we postulated that exogenous serum would result in a more differentiated phenotype of the VECs; thus, we investigated the same parameters with respect to this additive.

The morphology of the VEC cultures grown in PRF-SFM produced cells with a rounded shape and a large nuclear-to-cytoplasmic ratio, similar to that of native VECs derived from basal layers, such as might be seen on the vaginal smear from a woman not receiving hormone replacement therapy (Figure 1A). Occasional cells found among these cultures were substantially larger, with more pyknotic nuclei and abundant cytoplasm, more closely resembling native VECs from suprabasal layers. As cultures reached confluence, they gradually formed a cobblestone appearance.



A ***B***

Figure 1. ***A***: Vaginal epithelial cells grown in PRF-SFM, Wright-Giemsa stained ***B***: Vaginal epithelial cells grown in PRF-SFM and stimulated with 300 pg/ml 17-ß-estradiol for 72 hours, Wright-Giemsa stained

Treatment of the cell cultures with characterized fetal bovine serum for two or more days caused a change in morphology to a more differentiated appearance, resembling native VECs derived from intermediate or superficial layers. Treatment with 17-ß-estradiol also produced cells with a more differentiated morphology. The degree of these changes was dependent upon the concentration of 17-ß-estradiol, the degree of confluence at the time of hormonal application and the time of exposure. Although one goal of this proposal is to better define the relative effect of each of these factors and interrelationships between them, in the context of markers of differentiation, we noted several reproducible effects on cellular morphology. We tested 17-ß-estradiol concentrations from 5 pg/ml to 500 ng/ml. At concentrations over 400-500 pg/ml, there was a tendency to accelerated cell death, with increased vacuolization and granularity of cells still attached. The highest concentrations were essentially lethal to the cultures in a few days. If 17-ß-estradiol was applied to cultures at less than 20% confluence, this also tended to hasten cell death. Within a range of 100 to 250 pg/ml 17-ß-estradiol, applied at 60 to 80% confluence and kept constant, we observed the reproducible morphological changes towards a more differentiated phenotype, with

relatively consistent morphology throughout the culture. An example is shown in Figure 1B, above.

We conducted a few preliminary studies of progesterone stimulation of VECs in culture, adding progesterone at concentration ranges of 100 to 400 ng/ml. The effect of the hormone appeared to be an acceleration of cell death, even at relatively low doses. These studies will require further development and confirmation.

d. Problems: Last year we reported difficulties with time constraints related to use of the biological safety hood and inadequate incubator space. Thus, we purchased the new incubator mentioned in last year's report and also have made use of auxiliary hood space very kindly provided by Drs. Patton and Eschenbach.

3. Recommendations in relation to the Statement of Work

1. Primary bladder epithelial cells: We will continue to collaborate with Drs. Atala and Grady to cultivate bladder cell lines, establishing additional frozen stocks and cell pellets for further purification and characterization of GSLs.

2. Primary vaginal epithelial cells: In the next funding period, we will continue to characterize VECs, focusing on studying the expression of GSLs, especially those known to be involved in bacterial adherence (see below) as differentiation markers, but also investigating keratin expression. Our anticipated result will be to better establish our in vitro model of vaginal epithelium as means studying inhibitors of pathogen adherence to human vaginal epithelium. This tissue has not been thoroughly studied, but the oral epithelium has been more extensively characterized and appears to be histologically and biologically similar to the vaginal mucosa. Thus, we have looked to the literature in oral biology for paradigms of how to better characterize our system.

Optimizing cell culture methods: In the early phases of these studies, we explored various methods of optimizing the longevity and health of the cultured VECs. The techniques we developed allow us to maintain cultures from a single individual for up to three months, through careful management of expansion techniques and frozen stocks. However, throughout the time course of the proposed studies, we will continue to investigate methods of enhancing our culture technique. Most of our early work in testing culture "additives" was done using SFM, and some was done using a characterized SFM which was relatively unsupportive of these cells' growth at baseline. Thus, we will test methods which have been described as enhancing primary cultures of human oral and esophageal epithelium by investigators in our Comprehensive Oral Health Research Center, including (1) separately testing the effects of additives such as epinephrine, transferrin, or triiodothyronine; and (2) increasing the amount of bovine pituitary extract in an effort to increase the longevity of our cultures (23-25). Since oral epithelial cells reportedly grow best at calcium concentrations of 0.09 mM or less, we will also test varying the calcium concentration below our current level (0.09 mM), in the range of 0.05 to 0.09 mM (23-25).

Characterization of differentiation markers: To establish baselines for the expression of keratins and GSLs under the cell culture conditions described above, we will begin with a simple and systematic approach. We will compare various degrees of confluence, e.g. newly

seeded cultures; 60-80% confluent cultures; and 100% confluent cultures. We will test cells grown in SFM and PRF-SFM. Modifications of the medium described above that result in changes in growth and/or morphology may possibly be investigated. Lastly, we will study cells differentiated by the addition of serum.

Keratins: Based upon our discussions with Dr. Dale and our review of the literature, we have chosen to investigate the expression of a panel of keratins that are predicted to stain differentially in cultured VECs or tissue sections, and/or their expression may be regulated by estrogen exposure: (a) K19, expected to be found expressed in basal cells; (b) K14, likely to be found in cells of varying degrees of differentiation; (c) K13, expected to be expressed in suprabasal cells; (d) K10, described as appearing in a mosaic pattern in native vaginal epithelium (28, 29); and (e) K1, a keratin whose expression is regulated by estrogen in vivo and which has also been seen in a mosaic pattern in native tissue (28, 29). We will also stain for involucrin, anticipated to be found in superficial layers. Depending on the results of these studies, we may subsequently study the expression of K6 and K16, usually associated with rapidly turning over epithelia, wound healing, growth in cell culture, and some hyperproliferative disorders of epidermis, and normally found in palatal and gingival epithelia.

GSLs: Our preliminary data indicate that in cultured VECs, exposures resulting in a more suprabasal, differentiated morphology are associated with differential effects upon the expression of various families of GSLs. An exhaustive search for details of changes in GSL glycosylation that may occur at various stages of differentiation of VECs would be well beyond the scope of this proposal. However, we can reasonably screen for the expression of one member each of the lactoseries and neolactoseries families of GSLs. The bacterial overlay method we used to obtain our preliminary data, using GSLs extracted from two different individuals' cell lines, provides a broad probe for the expression of globoseries GSLs. To confirm these data, we will use the same approach on the remainder of the samples to be tested.

Antibodies: The names and sources of antibodies to keratins and involucrin are as follows: K14: LL001; monoclonal provided by Dr. Dale; K19: ICN; K13: AE8 from ICN or NCL-CK13 from Novocastra; K1: LHK1; provided by Dr. Dale; K10: NCL-CK10 Novocastra; Involucrin: SY5 Novocastra

Antibodies to GSLs: MAbs and epitopes against which these are directed are as follows: Lactoseries GSLs: we will test for Lewis_a or Lewis_b, depending on the source patient's Lewis type, using anti-Le_a and anti-Le_b (Gamma Biologicals); Neolactoseries: We will test for Le_y expression MAb ID4, available in our laboratory, courtesy of Dr. S.-I. Hakomori; Globoseries: E. coli R45 will be used in bacterial overlay assays and MAb ID4-E4 (27, 30) will be used in immunohistostaining and TLC immunostaining overlay assays on separated GSLs (17, 22), techniques that are in daily use in our laboratory.

Immunofluorescence in cultured cells: We will use the methods developed in Dr. Dale's laboratory (7, 8).

b. Task 2, Months 7 to 12: extraction and purification of GSLs from bladder and vaginal cell cultures

1. Experimental methods, assumptions and procedure

We continued extraction of GSLs from primary cultures of human bladder epithelial cells and VECs as needed for meeting the goals in the statement of work. The methods were unchanged from those described in our preliminary data: at each step, GSLs were chromatographed in various organic solvent systems and stained with orcinol (carbohydrate detection stain) to assess the purity of individual bands. In addition, HPTLC bacterial overlay procedures as described below were performed to identify and monitor the purification of GSLs of interest. Briefly, the GSL isolation and purification steps are as follows: cell cultures were maintained as described (13) then cells were trypsinized, pelleted, and washed, and the total GSLs were obtained by extracting the pellets with 10 volumes isopropanol:hexane:water (IHW; 55:25:20 by volume) with sonication in a warm bath and centrifugation at 2,500 RPM for 10 minutes. Some samples were subjected to a modified Folch procedure to obtain the upper and lower phase GSLs (15).

2. Results and discussion

For the bladder epithelial samples, our purification efforts are focusing on obtaining enough material for confirming the identity of the GSLs to which the representative E. coli isolates bound. Thus, we have expanded cultures of two bladder cell lines and obtained larger amounts of purified GSLs from these than in previous funding years. GSLs in these samples were separated into upper and lower phase mixtures in preparation for structural characterization techniques described below. Both samples have been subjected to various purification steps designed to remove contaminating free sugars and salts as well as phospholipids, cholesterol and triglycerides.

For our studies using vaginal epithelial cells, we have focused on obtaining adequate amounts of material from cell cultures exposed to the various conditions described in our studies regarding estrogen stimulation, e.g. growth in SFM vs PRF media. To date, we have analyzed 2 pellets. Data from these samples are shown below in the sections showing results of bacterial overlay studies, since we most often used this method to analyze the expression of relevant GSLs.

3. Recommendations in relation to the Statement of Work

We will not make major changes in technical approaches, but since GSL purification is extremely labor intensive and time consuming, we will tailor our efforts in this area to the needs dictated by our results. For example, we have designed the structural characterization experiments described below to deduce as much information about bacteria binding GSLs as possible from one GSL sample.

c. Task 3, Months 7 to 12: bacterial overlay assays

1. Experimental methods, assumptions and procedure

This assay involves separating GSLs on HPTLC plates and overlaying the plates with radiolabeled bacteria (3, 18). E. coli organisms were metabolically labeled with [^{35}S]methionine as previously described (3). S. saprophyticus organisms were also metabolically labeled with [^{35}S]methionine using a gonococcal labeling method (Mandrell, unpublished data) that we adapted for S. saprophyticus (Stapleton et al., manuscript in preparation). The organisms have a final specific activity of 0.01 cpm/organism, similar to the specific activity achieved using comparable methods for E. coli (3). Radioactive bacterial overlay assays were performed as previously described, with a HPTLC plate run in parallel for orcinol staining. For the bacterial overlay assays with E. coli, we have continued to primarily use metabolically labeled wild type E. coli R45 (3), which expresses the pap-encoded class II adhesin (19) and thus specifically recognizes globoseries GSLs. Overlays with S. saprophyticus have been performed using wild type isolate ST352, as in our preliminary data.

2. Results and discussion

GSL expression in cultured vaginal epithelial cells

Since the globoseries GLSs serve as binding receptors for uropathogenic E. coli in vaginal epithelium (30) and other tissues (16), we investigated the expression of this family of GSLs in cultured primary VECs grown under conditions which altered the adherence of these organisms. Methods were as described above in C.1.

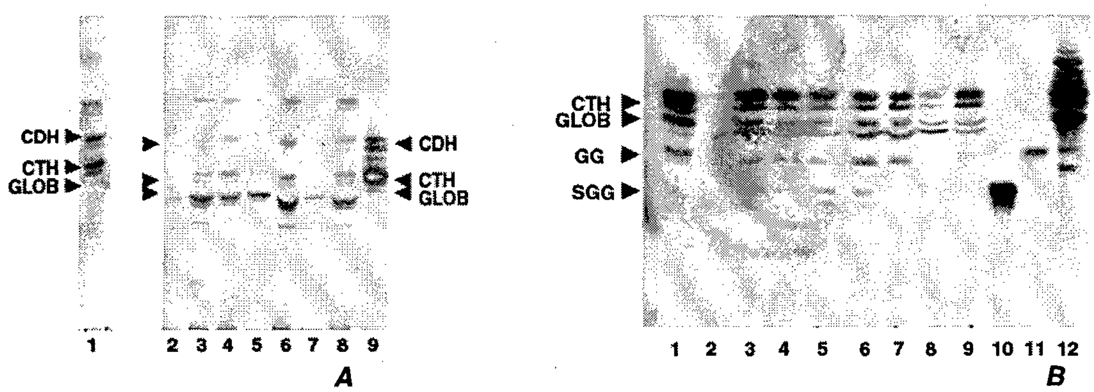


Figure 2. Binding of E. coli R45 to vaginal epithelial cell GSLs ***A***: Orcinol stained HPTLC plate. ***B***: Autoradiograph. Lane 1: V26 cells grown in SFM (note Lane 1A is taken from a different plate); Lane 2: V26 cells grown in SFM with PPPP, an inhibitor of GSL synthesis; Lane 3: V26 cells grown in SFM and changed to PRF-SFM for 24 hours prior to harvest; Lane 4: V26 cells grown in PRF-SFM and stimulated with 250 pg/ml 17-ß-estradiol for 24 hours; Lane 5: V26 cells grown in PRF-SFM and stimulated with 250 pg/ml 17-ß-estradiol for 77 hours; Lane 6: V31 cells grown in SFM; Lane 7: V31 cells grown in PRF-SFM; Lane 8: V31 cells grown in PRF-SFM and stimulated with 250 pg/ml 17-ß-estradiol for 24 hours; Lane 9A, GSL standards CDH, CTH, and GLOB,; Lane 9B: V27 cells grown in SFM; Lane 10B: SGG; Lane 11B: GG; Lane 12B: CTH and globoside (GLOB).

The results of these experiments paralleled those of our adherence studies (see below) with respect to effects of 17-ß-estradiol or phenol red exposure. The overall GSL content of the various cell pellets is reflected in the intensity of orcinol staining (for carbohydrate), as shown in Figure 3A. Short-term exposure (24 hours) to 17-ß-estradiol or phenol red increases overall GSL content, as compared with the results of growing cells in PRF-SFM. However,

the effect of 17-ß-estradiol treatment or growth in SFM had complex effects on the expression of globoseries GSLs, as reflected in the intensity of signal for the binding of E. coli R45, which recognizes these GSLs. Treatment with 17-ß-estradiol apparently induced or increased the expression of glycosyltransferases involved in the synthesis of more extended globoseries GSLs (5 or more carbohydrate residues), since the autoradiograph (Figure 3B) shows increased intensity of several slower migrating (lower) bands that bind E. coli R45 in 17-ß-estradiol-treated cell pellet extracts (Figure 3B). In addition, the hormone also increased expression of some non-globoseries, 4 or 5 sugar species, seen as dark, slowly migrating bands on the orcinol stained plate. With increased time of exposure to 17-ß-estradiol, the ratio of the amount of shorter-chained GSLs such as globotriaosylceramide (CTH; Gb3) to more extended globoseries GSLs appears not to change, but the intensity of binding of these GSLs by E. coli R45 is proportionally decreased. The effect of chronic growth in SFM is an increase in overall GSL content, but a proportional *decrease* in globoseries GSL content, as compared with cells grown in PRF-SFM. Thus, we have observed that 17-ß-estradiol has a differential effect on expression of families of GSLs in cultured VECs, and these findings parallel those of the adherence phenotype, which is a GSL-dependent process in VECs.

GSL expression in cultured bladder epithelial cells

Since data regarding E. coli-binding GSLs in cultured bladder epithelial cells are incorporated into a manuscript in preparation, we have not focused extensively on this area. Instead, we have worked on optimizing the overlay assays with S. saprophyticus, using GSL extracted from both bladder and vaginal epithelial cells. We have worked with various blocking conditions, buffers, concentrations of polyisobutylmethacrylate, and timing and have improved the assay, as shown below in Figure 3:

Figure 3. Binding of S. saprophyticus ST352 to epithelial cell GSLs. Lane 1: V17 primary vaginal cells; Lane 2: ASGM1; Lane 3: J82 bladder cells, lower phase; Lane 4: J82 bladder cells, upper phase; Lane 5: CWJ primary bladder cells

3. Recommendations in relation to the Statement of Work

1. Bacterial overlay assays involving E. coli will focus on confirming the data already generated and on further characterizing this model, e.g. through studies of the effects of estrogen on GSL expression and the like.

2. Significant progress has been made towards optimizing bacterial overlay assays using S. saprophyticus, but we still need to work on decreasing background. This next year, we will focus on confirming these results and expanding the studies to include other isolates, if time permits.

d. Task 4, Months 7 to 12: immunostaining assays

1. Experimental methods, assumptions and procedure

The non-radioactive method of performing HPTLC immunostaining assays mentioned in prior reports is now a routine procedure in the lab. We have continued working with Dr. Hakomori to produce fresh lots of the MAbs (TKH-7 and ID4) needed for immunostaining assays. We have supernatants of TKH-7 producing hybridomas that contain sufficiently high titers of specific MAb to complete our studies. However, we have had some difficulty in raising enough MAb ID-4 and have thus worked with Dr. Hakomori to optimize hybridoma cell culture conditions over the past few months.

2. Results and discussion

We have confirmed our preliminary data using MAb TKH-7 in immunostaining assays of GSLs extracted from bladder and VECs (data not shown). Since we have found that some cultures of primary VECs show staining with MAb TKH-7 using immunohistostaining techniques, we reasoned that the epitope recognized by this MAb could be expressed on a glycoprotein. Thus, we performed a pilot experiment using Western blot methods and MAb TKH-7 to stain protein extracts of the VECs. This experiment showed two reactive bands in the extracts. Because of time constraints and because this is not part of our original plans, we have not pursued this finding. However, if time permits in the last year of funding and it is logical to investigate this further, we will do so.

3. Recommendations in relation to the Statement of Work

1. We will to work with Dr. Hakomori to optimize hybridoma culture yields for ID-4 and then apply the MAb to studies as described.

2. Additional immunostaining assays will be performed as needed to confirm presumptive identification of GSLs in assay described above.

e. Task 5, Months 13 to 24: carbohydrate structural analysis; and

f. Task 6, Months 25 to 36: data analysis and publication

1. Experimental methods, assumptions and procedure

a. Definitive carbohydrate structural analysis: Full details of the methods used in purifying and performing definitive carbohydrate structural analysis of compounds mentioned below are provided in the appended manuscript (Stroud et al.)

b. New methods using exoglycosidases: We have recently begun pilot studies of using a new method of deducing carbohydrate structures among mixtures. The principle behind this method is as follows: when we are working with a mixture of structures purified from a source such as VECs, often we have tools for definitively identifying some of the bands seen when the mixture is separated on HPTLC, such as reactivity with specific MAbs directed against that carbohydrate structure. In addition, some of the structures of interest are members of families of GSLs, such as the globoseries, which differ from one another in structure only by one or two terminal sugar(s). Thus, we can identify some compounds for which we have specific MAbs by cleaving the relevant terminal sugar(s) with specific exoglycosidases and then using staining with MAbs, bacterial overlay assays, and assessment of relative mobilities on HPTLC to deduce the structures(s). An example of how we can take advantage of these structural relationships is shown below in Table 1. We have piloted this approach for identifying the globoseries GSLs that bind pap-adhesin expressing E. coli in our bladder cell extracts. Using Gb5 synthetically produced by Dr. Stroud (see below). we are using specific exoglycosidases to first cleave the terminal galactose to obtain Gb4 (globoside), followed by another enzyme to cleave terminal *N*-acetylgalactosamine from Gb4 to obtain Gb3, an epitope for which we have a specific MAb.

Table 1. Glycosphingolipid structures

Globoseries GSLs	**Structure** core: **Galα1-4 Galß1**-R
CTH (Gb3)	Galα1-4 Galß1-4 Glcß1-1cer
Globoside (Gb4)	GalNAcß1-3 Galα1-4 Galß1-4 Glcß1-1cer
Galactosyl globoside (Gb5)	Galß1-3GalNAcß1-3 Galα1-4 Galß1-4 Glcß1-1cer

2. Results and discussion

1. Results of carbohydrate structural analysis studies done with Dr. Levery are described below in the section related to Technical Objective 3.

2. Our pilot studies with exoglycosidase treatments using purified globoseries compound indicate that this approach is feasible, in that we have been able to achieve cleavage of both GB5 and Gb4, to a band co-migrating with Gb3. This result was recently achieved and required trials of various time, temperature and buffer conditions. The method requires further optimization but clearly is successful.

3. Recommendations in relation to the Statement of Work

1. We will continue work with Dr. Levery to perform carbohydrate structural analysis as appropriate.

2. The exoglycosidase method described above will be optimized and applied to our unknowns. We will perform immunostaining to identify the resulting Gb3 compound on HPTLC, definitively identifying the band co-migrated with standard Gb3.

2. A major effort will be made to complete several manuscripts in preparation, related to each of the technical objectives above.

2. Technical Objective 2

a. Task 1, Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures

1. Experimental methods, assumptions and procedure

We used the same methods described in our preliminary data, as follows: primary cultures of bladder and vaginal epithelial cells were maintained and utilized as described above. Bacterial adherence methods were as described in the preliminary data, using methods developed for use with cultured primary human bladder epithelial cells and exfoliated VECs (14). During the past year of funding, we used representative E. coli and Lactobacillus isolates that were used in some of our previous work: (1) E. coli R45 and IA2, two wild type uropathogenic isolates which express a Class II pap-encoded adhesin (3, 15), bind to the globoseries GSLs recognized by these adhesins in vitro (3), and reproducibly bind to exfoliated VECs or cultured primary human bladder epithelial cells (unpublished data); (2) Lactobacillus isolate AA68, an H_2O_2 producing strain which adheres avidly to exfoliated human VECs (14); and (3) Lactobacillus isolate AA52, an H_2O_2 negative strain that is poorly adherent to exfoliated human VECs (14). E. coli HB101, a non-adherent strain which does not express any known adhesins (11) served as a negative control, along with only a change of medium.

E. coli isolates were grown overnight on sheep blood agar at 37° C and lactobacilli were grown for two days on sheep blood agar in 5% CO_2. A full description of methods for selecting and growing these organisms is presented in an appended manuscript (Andreu et al.). An amount of organisms sufficient to produce the desired final concentration after washing was scraped from the plates. The organisms were resuspended in 1.0 mL of keratinocyte medium, incubated with primary cultures of human VECs at near confluence for 2 hours, washed repeatedly with PBS and fixed and stained using a Wright-Giemsa stain. The numbers of adherent organisms on the first 50 cells were counted by light microscopy (16).

Prior to the assays, the cells were maintained under various growth conditions to look for morphological evidence of alterations in the state of differentiation and to investigate if any of these changes could be associated with the number of adhering bacteria and/or the species

of bacteria for which adherence was affected. The following conditions were used for this pilot study: (1) SFM; (2) PRF-SFM; and (3) PRF-SFM, with 17-ß-estradiol (Sigma) added in four different concentration ranges for three different time periods. In addition, we added 10% fetal calf serum (Hyclone) to selected cultures for 72 hours to induce a suprabasal morphology (unpublished data).

In separate preliminary studies, we adapted a fluorescence-activated cell sorter (FACS) - based adherence assay we routinely use to study bacterial adherence to exfoliated native vaginal epithelial cells. E. coli IA2 and control non-adherent isolates were transformed with a plasmid expressing green fluorescent protein (GFP) and then used in standard bacterial adherence assays. Vaginal epithelial cells were grown in suspension and then subjected to our standard bacterial adherence assay conditions, using the GFP-transformed bacteria. The FACS then measures the amount of fluorescence of cells with and without adherent bacteria, and these data are expressed as green fluorescence index (GFI). The goal of these studies was to compare data from the living primary cultured vaginal epithelial cells with those obtained with the exfoliated vaginal epithelial cells (unpublished data).

2. Results and discussion

The results of our experiments with lactobacilli AA68 and AA52 paralleled our work with exfoliated VECs (14). The highly adherent strain AA68 adhered in very high numbers to cultured VECs, while adherence by strain AA52, which is nearly non-adherent to exfoliated VECs, was extremely low. It was not possible to accurately quantify the adherence of Lactobacillus strain AA68 using our visual counting method because of characteristic clumping and the high number of adhering organisms. To assess Lactobacillus adherence, we will apply a new computerized adherence counting method currently under development in the laboratory.

As compared with the Lactobacillus attachment, adherence by the E. coli isolates we selected is more readily quantified, and the mechanisms of adherence are better understood. In addition, we have extensive experience with using these isolates in adherence assays with primary cultured bladder epithelial cells. Thus, we conducted more pilot experiments with E. coli strains R45 and IA2, testing a total of 10 cell lines. In addition, we established that the adherence of E. coli expressing pap-encoded adhesins is dependent on GSL expression, based on treatment with a competitive inhibitor of GSL, PPPP (17). The results were dependent on the type of medium in which the cells were maintained as well as the concentration of 17-ß-estradiol added (Table 1). In SFM, adherence by either R45 or IA2 was essentially zero. In contrast, adherence of E. coli IA2 was much higher for cells grown in PRF-SFM, without the addition of 17-ß-estradiol, though these cells maintained a more basal appearing morphology (Figure 4). At this point, we have only used E. coli IA2 for adherence assays using PRF-SFM, although there is no reason to believe results would differ with E. coli R45.

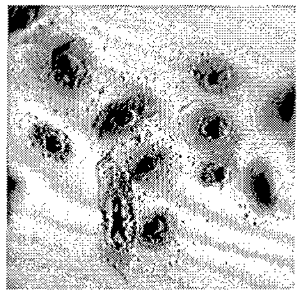


Figure 4. Vaginal epithelial cells grown in PRF-SFM, subjected to an adherence assay using E. coli IA2, and Wright-Giemsa stained

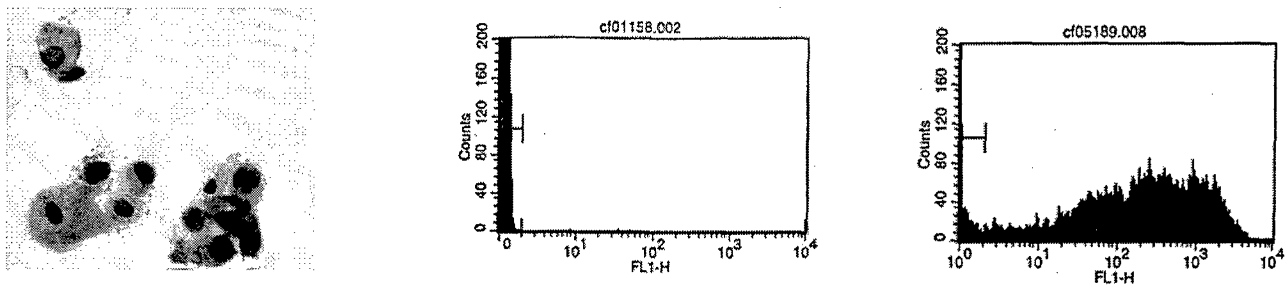
Our data suggest a complex relationship between the hormonal dose response, the time of exposure and the degree of maturation. When 17-ß-estradiol was added in a range of 100 to 400 pg/ml, the initial effect was an increase in the number of cells with a more mature, suprabasal appearance, after one to two days at the lower concentrations or one day's exposure to the highest concentration. For these mature cells, adherence of either E. coli strain was increased as compared with the number of organisms attaching to cells with basal morphology present in the same assay, or cells grown in SFM, which have a similar morphology. However, for cultures grown in parallel to those stimulated with 17-ß-estradiol as described above but allowed to continue for a total of three days of 17-ß-estradiol, bacterial adherence to these larger, more suprabasal appearing cells decreased towards the levels seen with unstimulated cells grown in SFM. Adherence to the basal-appearing cells in these cultures remained at minimal levels. These findings are reflected in a decrease in the mean adherence for cells with the highest level of and/or the longest time of exposure to 17-ß-estradiol (Table 1).

Table 1. E. coli IA2 adherence, bacteria per cell

		Concentration of exogenous estradiol, pg/ml			
		100	*200*	*300*	*400*
Exposure time, hours	24	3.8	3.7	11.5	5.4
	48	2.3	6.2	4.9	2.6
	72	1.5	3.5	1.4	3.6

Based on the results of these pilot experiments, we hypothesize that in our model, 17-ß-estradiol exposure for more than a brief period causes a downregulation of adherence by globoseries GSL-recognizing uropathogenic E. coli. This effect seems to be mirrored by chronic growth with phenol red, known to act through the estrogen receptor in other estrogen-responsive tissues (18). This would represent an example of a marker of differentiation being altered by this hormone in vaginal epithelium. In addition, the finding that exposure of VECs to estrogen in vitro might have a protective effect with respect to adherence of E. coli correlates with the clinical findings in post-menopausal women not receiving hormone replacement therapy. These women have an increased likelihood of vaginal colonization with E. coli (19, 20) and an increased risk of UTI (Fihn, et al., unpublished data), reversible with estrogen replacement.

The results of our experiments with VECs grown unattached and then subjected to standard adherence assays using E. coli IA2 with exfoliated native vaginal epithelial cells are shown below in Figure 5. Panel A shows data from a stained adherence assay and panels B and C show green fluorescence index (GFI) measured in the FACS when the same cells were subjected to this assay (unpublished data). These data confirm our findings with native exfoliated VECs. The GFI of cells to which E. coli IA2 has allowed to adhere is shifted to higher values, signifying adherence by the organism.



A B C

Figure 5. Adherence assays using unattached vaginal epithelial cells. Cells were grown unattached as described above and subjected to adherence assays using E. coli IA2 as described in the methods. **A**: modified Wright-Giemsa stain of adherence; **B**: GFI of vaginal epithelial cells alone; **C**: : GFI of cells with adherent E. coli IA2.

3. Recommendations in relation to the Statement of Work

1. The methods for additional bacterial adherence studies will be as described in the preliminary data, except a computer based method for analyzing adherence (already in use for other organisms in our laboratory) will be used for quantifying Lactobacillus (21). Cell culture and hormone exposure conditions will be determined based on the results of hormone exposure studies described above. We will begin by comparing adherence to cultures at maximally differentiated and undifferentiated states according to these findings, using the same organisms and controls utilized in our preliminary studies during the past year of funding.

2. If time permits, we will further develop studies of the adherence effects of exposing the vaginal epithelial cell cultures to progesterone.

3. Again, a major effort will be directed towards publication of the intriguing results described above.

b. Task 2, Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs

1. Experimental methods, assumptions and procedure

Methods used were as described in our preliminary data.

2. Results and discussion

In the early part of the past year, we confirmed some of our data demonstrating that the VECs are indeed epithelial cells and not fibroblasts. However, we realized that our data on estrogen stimulation of VECs suggest this model has considerable potential and that the epithelial biology of our culture system needs to be well characterized. We felt it was very important to identify and recruit collaborators who could help us perform relevant studies, many of which would involve immunocytochemical staining. Thus, we deferred much of this work for the last funding year in order to increase efficiency and avoid duplication of staining studies.

3. Recommendations in relation to the Statement of Work

Please see the descriptions of plans for more detailed characterization of cultured VECs described above in the recommendations for Technical Objective 1.

c. Task 3, Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays

1. Experimental methods, assumptions and procedure

Several inhibitors of GSL synthesis, such as 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP), an analog of glucosylceramide that competitively inhibits the synthesis of GSLs in living cells, have been synthesized (27) and some are available on the market. We performed pilot experiments to show that specific GSL synthesis inhibition was important in bacterial adherence to bladder epithelial cells by concomitantly performing bacterial adherence assays and extracting and quantifying the GSLs in treated versus untreated cultures. During the second year of funding, we began using a related compound, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) because it is the most active congener of this group of compounds but has effects similar to those of PDMP in tissues where it has been studied. This year, we confirmed experiments with this compound that were begun last year, using techniques described in our preliminary data. Briefly, VEC cultures were treated with PPPP and then one of the following assays were performed, as described above: (1) extraction of GSLs from cell pellets; (2) bacterial overlay assays using these cell pellets; and (3) bacterial adherence assays using intact, pre-treated cells.

2. Results and discussion

We confirmed the results of last year's studies, showing that PPPP drastically reduces or abolishes GSL synthesis in the cultured VECs, as evidenced by reduced or absent staining with orcinol and near abolition of bacterial binding using E. coli as a probe. We also repeated experiments in which PPPP treatment of intact cell cultures reduced the adherence of E. coli R45 from approximately 50 bacteria/cell to 0 bacteria/cell.

3. Recommendations in relation to the Statement of Work

As appropriate, we will include studies using this GSL synthesis protocol. Again, a major goal is publication of these results.

3. Technical Objective 3

a. Task 1, Months 24 to 42: synthesis of linear mimetic compounds

1. Experimental methods, assumptions and procedure; and
2. Results and discussion

After discussions with Dr.Toyokuni, we modified our approach for the past funding year, as described below, in order to produce more of the parent compounds with which Dr Toyokuni will work. Drs. Toyokuni and Stroud remain in frequent communication regarding this objective and will meet in November at a glycobiology meeting. The methods we will employ for the coming year, using the compounds synthesized by Dr. Stroud as starting material (see below) will be as described in our original statement of work.

Enzymatic synthesis of globoseries based GSL receptors

One of our primary goals is to chemically modify sialosyl galactosyl globoside (SGG) to identify the functional groups that give rise to its high affinity characteristics as a receptor for uropathogenic E. coli. To accomplish this, we pursued two separate methods to generate relatively large quantities of SGG for our chemical modification studies. Our first approach was based on an early report on the presence of SGG in chicken pectoral muscle. We detected a GSL that co-migrates with SGG in ganglioside fractions of chicken muscle and binds E. coli R45 using our HPTLC bacterial overlay assay. Our second approach is based on the partial enzymatic synthesis of fractions of chicken muscle and binds E. coli using our galactosyl globoside (Gb5), the immediate synthetic precursor to SGG in vivo. We detected relatively high levels of ß1-3 galactosyltransferase activity capable of catalyzing the biosynthesis of Gb5 using globoside (Gb4) as an acceptor substrate in two cell lines. While pursuing these strategies, we recently (confidentially) obtained cloned ß1-3 galactosyltransferase from a collaborator and assayed its activity using Gb4 as an acceptor substrate. The enzyme efficiently converted Gb4 in a reaction mixture containing UDP-galactose and $MnCl_2$ to a slower migrating GSL that co-migrated with a Gb5 standard when assessed by thin-layer chromatography. The enzyme was expressed as a soluble construct in insect cells (baculovirus system) purified to homogeneity and used to biosynthesize the putative Gb5. A large-scale purification of Gb4 from human erythrocytes was completed and used as the acceptor substrate. Gb5 was synthesized, purified and sent to Dr. Steve Levery at the University of Georgia for structural analysis. The structure of Gb5 was confirmed by ^{1}H-NMR as follows:

Galß1-3 GalNAcß1-3 Galα1-4 Galß1-4 Glcß1-1Ceramide

Once sufficient amounts of Gb5 are synthesized, we will sialylate the terminal galactose of Gb5 using CMP-sialic acid as the donor substrate and a commercially available α2,3 sialyltransferase to generate SGG for further studies.

In parallel with these studies, a rat liver α1-2 fucosyltransferase previously thought solely to utilize the ganglioside GM1 as an acceptor substrate was assayed using Gb5 as a substrate. Preliminary data suggests that this enzyme actually has a lower Km for Gb5 than GM1 and

catalyzes the biosynthesis of globo-H. The enzyme was expressed in COS-7 cells and a detergent extract of the cells was used as the enzyme source in a reaction mixture containing GDP-fucose, $MnCl_2$ and the detergent Triton CF-54. The final product will be confirmed by ^{1}H-NMR. Although this α1-2fucosyltranferase was identified and characterized over 15 years ago, the substrate Gb5 was not easily available until now. Sufficient quantities of SGG and globo-H will soon be available and we will have a complete panel of globo-series GSLs to perform additional E. coli binding studies.

Cloning of a potential human α1-4Galactosyltransferase

The Galα1-4Gal sequence found in all globo-series glycolipids is the minimum structural element required for binding of uropathogenic strains of E. coli expressing a Class II pap-encoded adhesin. This structure also forms the basis for the human P histo-blood group system. The gene encoding the glycosyltransferase responsible for catalyzing the synthesis of this linkage has yet to be identified. The LgtC gene product from N. gonorrhea is involved in the biosynthesis of lipidoligosaccharide (LOS) and encodes an α1-4Galactosyltransferase. Expressed sequence tags (ESTs) based on sequence similarity to the LgtC gene product were retrieved using the tBLASTn algorithm against a human EST database. EST sequences were aligned and overlapping ESTs were compiled to form a composite sequence with an open reading frame of 1113 bp. A Kyte and Doolittle hydropathy plot suggests this gene to encode a type-2 transmembrane protein characteristic of all known mammalian glycosyltransferases. A DXD motif found in all sugar nucleotide binding proteins is also present. Two clones containing this gene were purchased from Genome Systems and sequenced. One clone containing a 2.2Kb insert proteins is also present. Two clones containing this gene were purchased from Genome Systems and sequenced. One clone containing a 2.2Kb insert includes the start and stop codon plus 3' and 5' untranslated regions. It also contains a 500bp insert (possible intron) that disrupts the ORF. The second clone has an intact ORF but is missing the 5' end containing the start codon. The second gene fragment has been successfully cloned into pcDNA3 expression vector and restriction sites have been identified to allow us to splice in the 5' end of the first gene fragment to create the entire intact ORF. The pcDNA3 vector containing this gene will be used to transfect COS-7 cells and cell extracts will be used as the enzyme source for further characterization.

3. Recommendations in relation to the Statement of Work

1. SGG synthesis: Once sufficient amounts of Gb5 are synthesized, we will sialylate the terminal galactose of Gb5 using CMP-sialic acid as the donor substrate and a commercially available α2,3 sialyltransferase to generate SGG for further studies. The final product will be confirmed by ^{1}H-NMR.

2. Efforts to synthesize Globo-H needed for our further studies will continue and the final product will again be confirmed by ^{1}H-NMR.

3. We will continue studies directed towards determining if the potential cloned human α1-4Galactosyltransferase is the correct enzyme.

7. KEY RESEARCH ACCOMPLISHMENTS

- Establishment of in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells
- Characterization of glycosphingolipids (GSLs) expressed by these epithelial cells
- Characterization of bacterial adherence to these cells by organisms that are key uropathogens for healthy young women
- Establishment of the principle that GSLs are essential for the adherence of pap-adhesin-expressing uropathogenic E. coli in these systems
- Initiation of studies of the effects of exogenous estrogen on the expression of GSLs in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on bacterial adherence
- Cloning of a potential human α1-4Galactosyltransferase
- Enzymatic synthesis of globoseries based GSL receptors

8. REPORTABLE OUTCOMES

a. Manuscripts, abstracts and presentations (during the third year only)

Manuscripts

1. Stapleton, A. Host factors in susceptibility to urinary tract infections. In: Baskin LS. Ed. Advances in Bladder Research. New York, NY: Plenum Publishing Corporation, 1999.

2. Stapleton AE. Prevention of recurrent urinary-tract infections in women. Lancet 1999;353:7-8.

3. Schlager TA, Whittam TS, Hendley JO, Wilson RA, Bhang JL, Grady R, Stapleton AE. Expression of virulence factors among Escherichia coli isolated from the periurethra and urine of children with neurogenic bladder on intermittent catheterization. Pedi Infect Dis J 1999, in press

4. Hooton TM, Stapleton AE, Roberts PL, Winter C, Scholes D, Bavendam T, Stamm WE. Perineal anatomy and urine voiding characteristics in young women with and without recurrent urinary tract infections. Clin Infect Dis, in press.

5. Ummenhofer W, Stapleton A, Bernards C. Effect of Staphylococcus aureus bacteria and bacterial toxins on meningeal permeability in vitro. Reg Anesth and Pain Med 1999;24:24-29.

Abstracts

1. Gupta K, Hooton TM, Stapleton AE, Roberts P, Winter C, Deshaw N, Wobbe C, Fennell C, Denton A, Kahn JB, Stamm WE. Efficacy of self-diagnosis and self-treatment for management of uncomplicated recurrent urinary tract infections in women. Program and abstracts of the 36th annual meeting of the Infectious Diseases Society of America, abstract no. 39.

2. Lemley CA, Ballweber LM, Johnson ML, Kiselev AO, Stamm WE, Stapleton A, Lampe MF. Identification of putative Chlamydia binding proteins from HeLa cells and immortalized human cervical cells. The American Federation for Medical Research, Western section annual meetings. Carmel, California, February 1999. Abstracts.

3. Grady RW, Mitchell ME, Stapleton AE. High-throughput analysis of differential gene expression of in vitro urothelium exposed to uropathogenic Escherichia coli pDC-1. Program and abstracts of the 1999 annual meeting, American Urological Association, abstract no. 21.

4. Grady RW, Mitchell ME, Mahmoudi A, Stapleton AE. Expression array "gene chip" analysis of in vitro urothelium exposed to uropathogenic Escherichia coli pDC-1. Presented at the annual meeting of the American Academy of Pediatrics, 1999.

5. Patton DL, Agnew KJ, Meier A, Aura J, Hooton T, Stapleton A, Stamm WE. Influence of a single episode of intercourse on the vaginal flora and epithelium with and without condom use. Presented at the Infectious Diseases Society of Obstetrics and Gynecology, 1999.

6. Expression of virulence factors among Escherichia coli isolated from periurethra and urine of children with neurogenic bladder on intermittent catheterization. Schlager TA, Whittam TS, Hendley JO, Wilson RA, Bhang J, Grady R, Stapleton A. Proceedings of the Cyokines/Chemokines In Infectious Diseases Meeting, National Institutes of Health, Sepembert 8-10, 1999, Bethesda, Maryland (Poster #055)

7. Carriage of Escherichia coli and expression of virulence factors in urine and periurethra of patients with neruogenic bladder on intermittent catheterization. To be presented at the International Bladder Symposium, November 4-7, 1999, Washington DC

Manuscripts submitted

1. Hooton TM, Scholes D, Stapleton AE, Roberts PL, Winter C, Gupta K, Samadpour M, Stamm WE. A prospective study of asymptomatic bacteriuria in young sexually active women.

2. Eschenbach DA, Thwin SS, Patton D, Hooton TM, Stapleton A, Agnew K, Winter C, Meier A, Stamm WE. Influence of the normal menstrual cycle on vaginal examination and microflora characteristics.

3. Scholes D, Hooton TM, Roberts PL, Stapleton AE, Gupta K, Stamm WE. Risk factors for recurrent urinary tract infection in young women.

Presentations and Meetings

1. Invited speaker and Scientific Committee, 1999 International Bladder Symposium

2. Invited speaker, University of Washington Science in Medicine New Investigator series, 1999-2000

3. Enteric Pathogens and Host Interaction; joint course conducted by the University of Washington and UNAM, Mexico City, November 2-7, 1998

Invited review in preparation

1. Molecular strategies for the prevention and treatment of urinary tract infections. Molecular Medicine Today.

2. Drug-resistant urinary tract infections. Current Practice of Medicine.

Awards

Masaaki Ohkoshi Award, International Congress of Chemotherapy, London, 1999
(given for publication of the manuscript entitled, "The globo-series glycosphingolipid sialosyl galactosyl globoside is found in urinary tract tissues and is a preferred binding receptor in vitro for uropathogenic Escherichia coli expressing pap-encoded adhesins", Stapleton AE, Stroud MR, Hakomori SI, Stamm WE, Infect Immun 1998;66: 3856-3861, judged to be most meritorius paper in the UTI field since the last international meeting).

Development of cell lines

Primary cultures of vaginal epithelial cells derived from individuals have been developed for the purposes of these studies. However, these cells have not been provided to other investigators as a "reagent" and are not suitable for an application of that nature, such as submission to a cell banking facility.

9. CONCLUSIONS

Our initial years of funding were focused on refining the techniques we developed for culturing primary vaginal epithelial cells and on adapting Dr. Atala's methods for culturing primary human urothelial cells to our model system. We have now established these in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells for the identification, purification and structural characterization of E. coli- and S. saprophyticus-binding glycosphingolipid moieties. We have completed studies characterizing bacterial adherence to these cells by organisms that are key uropathogens for healthy young women, E. coli and S. saprophyticus. We have also established that GSLs are essential for the adherence of pap-adhesin-expressing uropathogenic E. coli in these systems. Most recently, we have initiated studies of the effects of exogenous estrogen on the expression of GSLs in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on bacterial adherence. We have established several new collaborations that will allow us to expand and advance our studies related to epithelial biology, especially those of the vaginal epithelium. Establishing our model of the vaginal epithelium opens opportunities for studying numerous aspects of women's urogenital health, including testing vaginal products, probiotics, and contraceptives; understanding protective roles of organisms in the normal

flora; and studying the cellular effects of hormone replacement therapy on a key target tissue, the vagina.

The globoseries GLSs have not been thoroughly studied in normal adult tissues. Thus, the new approaches taken by Dr. Stroud in undertaking the enzymatic synthesis of globoseries GSLs and cloning of a potential human α1-4Galactosyltransferase have yielded basic findings that are important in the field of glycobiology, apart from their relevance to this work.

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11. APPENDICES

Appendix A: reprint of Stroud MR, Stapleton AE, Levery SB. The P histo-blood group related glycosphingolipid sialosylgalactosylgloboside as a preferred binding receptor for uropathogenic Escherichia coli: Isolation and structural characterization from human kidney. Biochemistry 1998;37: 17420-17428

Appendix B: reprint of Andreu A, Stapleton AE, Fennell CL, Hillier SL,Stamm WE. Hemagglutination, adherence and surface of vaginal Lactobacillus species. J Infect Dis 1995; 171: 1237-1243.

Appendix C: reprint of Stapleton, A. Host factors in susceptibility to urinary tract infections. In: Baskin LS. Ed. Advances in Bladder Research. New York, NY: Plenum Publishing Corporation, 1999.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

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